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(71) Applicant(s)

Merck & Co Inc
(Incorporated in USA - New Jersey)
P O Box 2000, 126 East Lincoln Avenue, Rahway,
New Jersey 07065-0900, United States of America

(72) Inventor(s)

Gerard F Hickey

(74) Agent and/or Address for Service

I J Hiscock
Merck & Co. Inc, European Patent Department,
Terlings Park, Eastwick Road, HARLOW, Essex,
CM20 2QR, United Kingdom

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(56) Documents Cited

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(54) Abstract Title

Method for increasing muscle mass in animals

(57) A method for increasing the muscle mass in animals, such as cow, sheep, pig and chicken, comprises (a) administering a vaccine which will promote the production of anti-myostatin (i. e., anti- growth differentiation factor 8 or GDF-8) antibodies, or (b) providing the animal with an immunoneutralising amount of anti-myostatin antibodies. Myostatin, a member of the transforming growth factor (TGF) superfamily of proteins, is thought to exert a negative control on the amount of skeletal muscle mass in an animal. The use of a vaccine or antibodies to myostatin allows one to increase the skeletal muscle mass in domesticated animals and thus increase their value as food sources. The vaccine may be a hapten-carrier protein conjugate in which the hapten is an epitope of myostatin, particularly from the functional domain at the C-terminus, or it may be a fusion protein comprising such an epitope fused to a carrier protein. The fusion protein product is obtained using standard recombinant DNA procedures using E Coli as host. The vaccine is preferably administered in a formulation also containing an adjuvant such as an aluminium salt (AlPO₄) or an oil-in-water emulsion such as vitamin-E acetate solubilisate. Immunoneutralisation of myostatin may occur after a single dose or a once-yearly dose may be applied. Immunoneutralisation may also be induced in pregnant animals resulting in transplacental transfer of anti-myostatin antibodies to the foetus and consequent increased muscle mass in the offspring.

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TITLE OF THE INVENTION

METHOD FOR INCREASING MUSCLE MASS IN ANIMALS

CROSS REFERENCE TO RELATED APPLICATIONS

- 5 This application is based on and claims priority from provisional application number 60/073,438 filed February 2, 1998, which is hereby incorporated by reference in its entirety.

SUMMARY OF THE INVENTION

- 10 The present invention relates to a method for increasing animal muscle mass by immunoneutralizing myostatin.

BACKGROUND OF THE INVENTION

- The protein myostatin (also known as
15 growth/differentiation factor-8 or GDF-8), a member of the transforming growth factor superfamily, is synthesized by skeletal muscle and has been hypothesized to regulate the amount of skeletal muscle mass in a negative manner. The hypothesis is based upon the findings that there is an associated increase in the amount of skeletal muscle in myostatin
20 gene knock-out mice as well as two breed of cattle, the Belgian Blue and Piedmontese, where a non functional myostatin protein is expressed (see for example, McPherron and Lee, Proc. Natl. Acad. Sci. USA, 1997, 94:12457-12461; Grobet et al, Nature Genetics, Sept. 1997, 17:71-74; McPherron et al, Nature, 1 May 1997, 387:83-90; and Kambadur et al,
25 Genome Res., 1997, 7:910-915). Myostatin genes from different species have been cloned and sequenced and have been found to be highly conserved across species. Many myostatin gene sequences have been deposited in the GenBank database, which is publicly available.

- Myostatin is composed of a secretory sequence (amino acids
30 1 - 262), a proteolytic processing site (amino acids 263-266) and the functional domain at the C-terminus (amino acids 267-375), which has a highly conserved tertiary structure due to the presence of multiple cysteine residues. The identification of myostatin as a negative muscle growth regulator allows for potential manipulation of muscle

development in livestock, including immunoneutralization of myostatin according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention is directed towards inducing an active immune response against myostatin that will result in the production of neutralizing antibodies against myostatin. Animals immunized against myostatin will be immunologically released from myostatin influence resulting in increased skeletal muscle mass in the
10 vaccinated animals.

 Accordingly, one aspect of the present invention provides a method for increasing muscle mass in an animal which comprises (a) administering to said animal a vaccine capable of eliciting anti-myostatin antibodies; or (b) providing to said animal an
15 immunoneutralizing amount of anti-myostatin antibodies.

 As used herein, the term "animal" includes livestock such as cattle, pigs, sheep, and the like, as well as poultry such as chickens, turkeys, ducks, geese, and the like.

 The term "vaccine" includes hapten-carrier protein
20 conjugates in which the hapten is an epitope of myostatin, particularly from the functional domain of myostatin, or a fusion protein containing such an epitope and a carrier protein.

 Two alternate approaches may be used to identify immunoneutralizing epitope sequences. In the first approach
25 overlapping peptide sequences based on the myostatin functional domain are synthesized such that the peptides are approximately 10 amino acids in length and overlap adjacent peptides by approximately 5 amino acids. The functional domain of myostatin therefore will result in 22 discrete peptides of 10 amino acids each.

30 A second approach is to identify potential epitopes based upon a three dimensional molecular modeling of myostatin. The presence of 9 cysteine residue will result in a rigid tertiary structure. This can then be compared to the three dimensional structure of other members of the TGF superfamily such as TGF beta who also have

multiple cysteine residues. Those regions of myostatin that are exposed or, alternatively that are similar in location to regions of ligands in the TFG superfamily known to be involved in ligand receptor coupling, would be identified and be evaluated as potential epitopes.

5 The candidate epitope sequences identified may be synthesized on an peptide synthesizer according to generally known procedures. If no cysteine is present in a particular peptide sequence, then a terminal cysteine may be added to the individual sequence to provide a means for conjugation to the carrier protein.

10 Carrier protein may be any known in the art for the purpose of increasing immunogenicity of the hapten. Suitable examples include, but are not limited to diphtheria toxoid, tetanus toxoid, KLH, ovalbumin, Pseudomonas exotoxin and variants thereof, leukotoxin, fimbrial subunit protein, helper T-cell epitopes such as those disclosed in
15 WO94/25060, and the like.

 Methods for conjugating a peptide to a macromolecule are well known in the art, and are applicable to the preparation of the vaccine of the present invention. Generally, the myostatin epitope and the carrier protein are linked through a cross linking reagent such as
20 SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), glutaraldehyde, iminothiolane, N-acetyl-homocysteine thiolactone, bromoalkanoic anhydrides, maleimido-benzoyl-N-hydroxy-succinimide ester, 3-maleimidopropionic acid N-hydroxysuccinimide ester, and the like. Essentially any method where nucleophilic and electrophilic groups are
25 provided on the reacting partners is sufficient to achieve linkage of peptides. The preferred cross linking agent for the present invention providing an electrophilic partner for the coupling reaction are active esters of maleimidoylalkanoic acids, and bromoalkanoic anhydrides. Preferred cross linking partners providing a nucleophile for the
30 coupling reaction are N-acetyl homocysteine thiolactone and imino thiolactone.

 The vaccine of the present invention may also be fusion proteins of myostatin epitope and a carrier protein. Such fusion proteins contain contiguous sequences of the constituent proteins or peptides.

The fusion proteins are preferably manufactured through expression of recombinant DNA sequences, and the manufacturing processes are well known in the art.

5 The DNAs used in the practice of the invention may be natural or synthetic. The recombinant DNA segments containing the nucleotide sequences coding for the embodiments of the present invention can be prepared by the following general processes:

(a) A desired DNA sequence is cut out from a plasmid in which it has been cloned, or the sequence can be chemically
10 synthesized;

(b) Then a second DNA sequence, the targeted DNA sequence, is cleaved at a specific location; and

(c) The desired DNA sequence is then brought into alignment with the cut in the targeted DNA sequence and the two
15 sequences are connected together through standard ligation procedures. The resulting recombinant gene is ligated down stream from a suitable promoter in an expression vector.

Techniques for cleaving and ligating DNA as used in the invention are generally well known to those of ordinary skill in the art
20 and are described in Molecular Cloning, A Laboratory Manual, (1989) Sambrook, J., et al, Cold Spring Harbor Laboratory Press.

As the promoter used in the present invention, any promoter is usable as long as the promoter is suitable for expression in the host used for the gene expression. The promoters can be prepared
25 enzymatically from the corresponding genes, or can be chemically synthesized.

Conditions for usage of all restriction enzymes are in accordance with those of the manufacturer, including instructions as to buffers and temperatures. The enzymes may be obtained from New
30 England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim and Promega.

Ligations of vector and insert DNAs are performed with T4 DNA ligase in 66 mM Tris-HCl, 5 mM MgCl₂, 1mM DTE, 1mM ATP, pH

7.5 at 15°C for up to 24 hours. In general 1 to 200 ng of vector and 3-5x excess of insert DNA are preferred.

Selection of *E. coli* containing recombinant plasmids involve streaking the bacteria onto appropriate antibiotic containing LB agar plates or culturing in shaker flasks in LB liquid (Tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.4) containing the appropriate antibiotic for selection when required. Choice of antibiotic for selection is determined by the resistance markers present on a given plasmid or vector. Preferably, vectors are selected by ampicillin.

Culturing *E. coli* involves growing in Erlenmeyer flasks in LB supplemented with the appropriate antibiotic for selection in an incubation shaker at 250-300 rpm and 37°C. Other temperature fro 25 - 37 C could be utilized. When cells are grown for protein production, they are induced at A560 = 1 with IPTG to a final concentration of 0.4 mM. Other cell densities in log phase growth can alternatively be chosen for induction.

Harvesting involves recovery of *E. coli* cells by centrifugation. For protein production, cells are harvested 3 hours after induction though other times of harvesting could be chosen.

In the present invention, any vector, such as a plasmid, may be used as long as it can be replicated in a prokaryotic or eukaryotic cell as a host.

The host cell of choice is *E. coli* HB101. However, a number of other *E. coli* strains would be suitable. For routine cloning, *E. coli* strain DH5a(BRL) can be used.

In the present invention, the fusion proteins can be separated and purified by appropriate combinations of well-known separating and purifying methods. These methods include methods utilizing a solubility of differential such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a

difference in hydrophobicity such as reverse-phase high pressure liquid chromatography, methods utilizing a difference in isoelectric point, such as isoelectric focusing electrophoresis, and methods using denaturation and reduction and renaturation and oxidation.

5 The vaccines of the present invention are preferably used in a formulation comprising an adjuvant. Suitable adjuvants are any of those substances recognized by the art as enhancing the immunological response of an animal to an immunogen without causing an unacceptable adverse reaction, and include aluminum compounds such as $\text{Al}(\text{OH})_3$, AlPO_4 , $\text{Al}_2(\text{SO}_4)_3$, water-in-oil emulsions such as
10 Incomplete Freund's Adjuvant (IFA), Bayol F® or Marcol F®, vitamin-E acetate solubilisate, saponins, muramyl dipeptides in an appropriate solvent such as squalane or squalene, or an adjuvant system comprising squalane, Tween® 80 and Pluronic® L121.

15 The dose/time adjustments associated with the use of vaccines of the present invention can vary considerably and will depend on a variety of factors such as the species of animal to be treated, the particular epitope and/or carrier used, the adjuvant, the age of the animal, and the desired outcome of vaccination. In general, the vaccine
20 is administered by subcutaneous or intramuscular injection into an animal at a dose of about 0.1 μg to 10 mg myostatin epitope-carrier protein per dose. A single dose of the immunogenic carrier system of the present invention may be all that is required to achieve
25 immunoneutralization of myostatin, but multiple doses spaced at one to six week intervals, or yearly vaccination for the life of an animal are alternative immunization schemes.

 In addition to immunoneutralization of myostatin to achieve increased muscle mass in the immunized animal, immunoneutralization can be induced in the pregnant mother resulting
30 in transplacental transfer of neutralizing antibodies to the fetus or in the colostrum to the neonate resulting in increased muscle mass in the offspring. Post natal immunization may also be carried out. In poultry, immunization of the layer hen, the egg or the neonate may be accomplished.

Vaccines of the present invention may be evaluated in rats as follows. The vaccine candidates are prepared in complete and/or incomplete Freund's adjuvant (CFA or IFA) and injected intramuscularly or subcutaneously into rats at a concentration of 0.1 to 10 mg/rat. Two or more separate injections are administered 4 weeks apart which should result in the production of high anti-peptide titers 2 weeks following the final booster. To determine the period during which inhibition of myostatin results in increased muscle mass, vaccination regimen is carried out in rats to ensure high anti-peptide titers are present at the following times, pregnant rat 1, 7 or 14 days post breeding, neonatal rats at 1, 7, 14, 21, 28, 35, 42, 49 or 56 days of age. Sacrifice and detailed necropsies are conducted at any time up to day 60 of life. The vaccinated rats are compared to adjuvant and non injected controls groups for any change in skeletal muscle mass.

The peptide vaccines are ranked according to their potency in increasing skeletal muscle mass in rats. These vaccines are then be further evaluated in the pig. Vaccination regime is carried out in pregnant pigs to ensure high anti-peptide titers are present through out gestation. In addition the effect of vaccination on growing pigs is evaluated by vaccinating groups starting at 1,4,8,12, or 16 weeks of age and with monthly boosters as required. Animals are slaughtered at 20-24 weeks of age and the change in skeletal muscle mass determined.

Similar approaches to that outlined for pigs could be conducted for cattle and other species of interest with the obvious modifications in the number and timing of vaccinations required based upon the varying life span of the individual species.

For poultry, in addition to injection of the layer hen and chick, the egg could also be injected with the epitope. A single vaccination at 1 mg/animal is carried out, and the effect of in ovo injections evaluated on days 1,5,10,15 of incubation and at 1,7,14 and 21 days post hatching.

WHAT IS CLAIMED IS:

1. A method for increasing muscle mass in an animal which comprises: (a) administering to said animal a vaccine capable of
5 eliciting anti-myostatin antibodies; or (b) providing said animal an immunoneutralizing amount of anti-myostatin antibodies.
2. A method of Claim 1 wherein said vaccine comprises
10 a myostatin epitope - carrier protein conjugate.
3. A method of Claim 2 wherein said myostatin epitope is selected from the functional domain of myostatin.



Application No: GB 9902041.4
Claims searched: 1-3

Examiner: Dr Lawrence Cullen
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Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

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Other: Online: CAS ONLINE; EPODOC, PAJ, WPI, STN INDEX

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X, E	WO 99/06559 A1 (JOHNS HOPKINS) see line 14, page 19 to line 23, page 24; see claim 39	1-3
X, P	WO 99/02667 A1 (UNIVERSITY OF LIEGE) see lines 1-5, page 1; lines 8-18, page 6; lines 4-18, page 8; line lines 30-34, page 27;	1-3
X, P	WO 98/33887 A1 (JOHNS HOPKINS) see lines 16, page 2 to line 3, page 3; line 10, page 18 to line 22, page 19; lines 1-13, page 37; lines 12-25, page 46; see claim 24-29.	1-3

X Document indicating lack of novelty or inventive step
Y Document indicating lack of inventive step if combined with one or more other documents of same category.
& Member of the same patent family

A Document indicating technological background and/or state of the art.
P Document published on or after the declared priority date but before the filing date of this invention.
E Patent document published on or after, but with priority date earlier than, the filing date of this application.